

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	September 1997	Annual (1 Sep 96 - 31 Aug 97)	
4. TITLE AND SUBTITLE		5. FUNDING NUMBERS	
Oligosaccharide Markers for Prognosis of Low-Risk Breast Cancer		DAMD17-94-J-4363	
6. AUTHOR(S)			
David E. Pettijohn, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)		8. PERFORMING ORGANIZATION REPORT NUMBER	
University of Colorado Health Sciences Center Denver, Colorado 80262			
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			
11. SUPPLEMENTARY NOTES			
19980212 068			
12a. DISTRIBUTION / AVAILABILITY STATEMENT			
Approved for public release; distribution unlimited			
13. ABSTRACT <i>(Maximum 200)</i>			
<p>Progress to date has included the determination of the occurrence of several different cell surface oligosaccharides and proteases on sections from 181 different ductal breast carcinomas. Monoclonal antibodies specific for the blood group related oligosaccharides Le^a, Le^x, sialyl- Le^a, Le^y, Tn, Sialyl Tn and extended Le^a- Le^x were used to define the cell surface oligosaccharides and both immunofluorescence techniques and immuno streptavidin-biotin techniques were used to evaluate the fraction of tumor cells that are positive and the relative amount of reaction product as measured using an image processing system. The preliminary analysis of the available data has shown that the extended Le^a-Le^x oligosaccharide is a statistically significant indicator of poor prognosis following surgical removal of small, node-negative ductal breast carcinomas. cDNA libraries constructed from cells that express Le^a- Le^x have been screened and selected for cDNAs that alter the expression of this oligosaccharide in cancer cells. Three candidate genes have been identified and preliminarily characterized.</p>			
14. SUBJECT TERMS		15. NUMBER OF PAGES	
Breast Cancer		prognosis, oligosaccharides, carbohydrates, glycoproteins immunofluorescence, monoclonal antibodies	
		8	
16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT		18. SECURITY CLASSIFICATION OF THIS PAGE	
Unclassified		Unclassified	
19. SECURITY CLASSIFICATION OF ABSTRACT		20. LIMITATION OF ABSTRACT	
		Unlimited	

AD _____

GRANT NUMBER DAMD17-94-J-4363

TITLE: Oligosaccharide Markers for Prognosis of Low-Risk Breast Cancer

PRINCIPAL INVESTIGATOR: David E. Pettijohn, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DATA QUALITY EVALUATION

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Dane E. Belljoh 9/26/97

TABLE OF CONTENTS

ANNUAL REPORT FOR GRANT NUMBER DAMD17-94-J-4363

Front Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Conclusions	7
References	7
Appendix	8
Bibliography	8

INTRODUCTION

Breast cancer patients who have a small node-negative ductal carcinoma generally have a favorable prognosis (see for review ref. 1). During a 5-10 year period after surgery, relapse occurs in less than 20% of these so called low-risk patients. However, managing such patients can be difficult, as there is no clear way of identifying the 20% who will relapse. The patients who could benefit most from adjuvant chemotherapy cannot be identified and equally important, the patients who don't require post-surgical adjuvant therapy cannot be unambiguously identified. The purpose of the present research is to devise an approach for identifying the low-risk patients who are at risk.

The general goal of these studies is to determine if there are specific combinations of oligosaccharide markers and other makers on breast cancer cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. Useful prognostic markers identified from these studies would then be combined with other known prognostic markers in an attempt to assemble a set of markers which could indicate with highest specificity and sensitivity the patients who are at greatest risk for relapse. The studies are also intended to identify a new glycosyltransferase activity that seems to be expressed in certain carcinomas and is correlated with poor prognosis. This identification would open the way for new approaches to studying the biological effects of the significant oligosaccharides that are correlated with poor prognosis.

We are studying a large group of breast tumor specimen obtained from a collection of the Danish Breast Cancer Cooperative Group which is a nationwide surveillance and research program (2). All specimen are from women who had low-risk node negative ductal breast carcinomas and who had surgery 5-20 years previously and who have been closely followed since surgery. None of the women had chemotherapy, so that the prognosis is unaffected by other post-surgical interventions. A panel of well characterized monoclonal antibodies with known specificity for specific oligosaccharides is employed to define the cell surface oligosaccharides, proteolytic activities (such as Cathepsins) and protease inhibitors associated with the tumor cells. After completing the analysis, the relapse history of the patients will be compared with the different molecular markers using Cox's proportional hazards model (3) to identify statistically significant independent markers of prognosis. It will then be possible to select different combinations of markers to attempt to improve specificity and sensitivity by using a panel of prognostic markers.

Additional related research is seeking to identify the glycosyltransferase activities that are abnormally expressed in breast cancer cells that lead to aberrant expression of specific marker oligosaccharides. Here we are attempting to clone cDNAs recognizing genes that are expressed in cells overexpressing the Le^a-Le^x oligosaccharide, which is the best prognostic indicator which we have identified. We are also beginning studies of the effects of Le^a-Le^x cell-cell interactions in carcinomas.

This research is still in progress and was planned to be in progress at this stage. Therefore conclusions and detailed summaries of the data to date are premature. However the preliminary review of the data provided below indicates that there could be a statistically significant association of the Le^a-Le^x oligosaccharide and poor prognosis of low-risk ductal breast carcinomas.

BODY

We continue to use the panel of monoclonal antibodies (Mabs) specific for the designated oligosaccharides and in the last year have applied the complete panel to multiple paraffin sections of an additional 72 tumor specimens and have started the sequence on an additional 23 specimens. Thus within a few months we will have completed the immunohistochemical analysis of in total 181 low-risk node negative tumors. This is somewhat less than the original number stated in our initial proposal and we now believe that we will not be able to obtain valid results from the 79 additional specimen which were described in the initial proposal. Our Danish colleagues in Odense have informed us that they have had to remove some of the specimens from the study because: 1) some specimen were nearly exhausted and when remnants were

sectioned only surrounding normal tissue was found in the sections; 2) After careful review of the clinical data, it was found that several patients had received chemotherapy either before or after surgery and therefore should not have been included in this study. These had been missed in an earlier review and, since our study is exclusively of patients who had no chemotherapy, these had to be eliminated. 3) Two specimens were eliminated because they were found to be from patients who were node-positive and this had not been previously noted on the clinical data. Thus their previous inclusion in our node-negative set was a mistake.

As in the previous year, we used double-label immunofluorescence microscopy techniques that apply fluorescein and rhodamine conjugated antibodies simultaneously so that the distribution of two different oligosaccharides can be simultaneously determined in the same tumor section (4,5). The Quantimet 500+ Image Processing System was used to analyze fluorescence images and to define both the fraction of tumor cells that are positive, (above a defined baseline), and the intensity of the reaction relative to positive and negative control cells that are processed at the same time. The fraction of positive tumors cells and the relative amounts of each cell surface components on the tumor cells is therefore determined.

As reported in the last progress report, we continue to observe significant heterogeneity for these oligosaccharides among the cells in certain tumors. For example Mab 43-9F recognizing the extended $\text{Le}^a\text{-Le}^x$ oligosaccharide reacts with nearly 100% of the cells of a few breast carcinomas and about 30% of other carcinomas are completely negative, but the majority of the carcinomas have a fraction of cells that are positive, ranging from 1 to 100 % of the cells in a section. As noted in the last progress report and recently published (6), we are now beginning to understand some of the reasons for this. We found that the expression of many oligosaccharides on the cell surface of cancer cells is dependent on the interactions with adjacent cells. For example, some tissue culture cells such as NU6-1 cells (that we are using to clone the genes coding for critical glycosyltransferases -see below) express large amounts of cell surface $\text{Le}^a\text{-Le}^x$ oligosaccharide when the cells grow touching neighbors in small colonies or in confluence layers, but express no detectable $\text{Le}^a\text{-Le}^x$ when cells are attached to substrata and growing without close neighbors. Other cell surface oligosaccharides appear when cells grow at low densities, but extinguish or are less plentiful when cells reach confluence.. Thus it seems that the prognostic markers that we study inform us about more than just the status of the individual cells in tumors, but also convey information about the cell-cell signaling within the tumor.

It may be important that our preliminary studies of patients who relapsed with ductal breast carcinomas have indicated that there is a correlation between the fraction of $\text{Le}^a\text{-Le}^x$ positive tumor cells and the period before relapse occurs. Patients with higher fractions of positive cells tended to relapse in shorter periods of time after surgery.

There is ongoing statistical analysis at the Biostatistics Core Laboratory of the University of Colorado Cancer Center of the 181 tumor specimen that have been completed or in progress. We are analyzing both single markers, multiple markers in combinations, and attempting a protocol for the analysis of the ratios of makers in attempts to sharpen prognostic indications of the multiple markers. As noted in the last progress report, the preliminary analysis of the $\text{Le}^a\text{-Le}^x$ marker alone showed statistically significant ($P<.005$) correlation with poor prognosis. The statistical analysis is using the proportional hazards model of Cox (6). We anticipate that we will be able to complete on time Tasks 1,2, and 3 that cover this area of our research, but with the somewhat fewer tumors , as discussed above.

We continue to make progress with the project designed to clone cDNAs specifying the glycosyltransferase required to extend Le^a into $\text{Le}^a\text{-Le}^x$ oligosaccharides (Task #5), however this cloning is not yet completed. As noted in the last report the p-bluescript cDNA library made from total mRNA of the human lung cancer cell line NU6-1 (which overexpresses the $\text{Le}^a\text{-Le}^x$ oligosaccharide (4) was subjected to subtractive hybridization against a similar library made from cDNA homologous to mRNA of the NU6-1 variant clone NE-18 (that makes no detectable $\text{Le}^a\text{-Le}^x$). This yielded 3 clones that were of interest among the many that were screened. Two clones have cDNA sequences of unidentified genes, and as previously noted the third clone has the DAF gene (Decay Accelerating factor)(7-9). These then

correspond to mRNA plentiful in a cell line that makes Le^a-Le^x and absent in a close variant cell line that makes no detectable Le^a-Le^x. These genes have been transfected into several different cell lines and we have seen in several cases dramatic changes in the expression of cell surface oligosaccharides. Currently, we are cloning these genes into a pCMVLac I vector system which places the cDNA under the control of an IPTG inducible promoter. With this system it should be possible to switch on and off the expression of the genes and thus investigate in detail the effects of these genes on the expression of Le^a-Le^x and other oligosaccharide tumor markers.

A recent collaborative project with Drs U. Engel and E. Hage of the State University Hospital in Copenhagen has shown that there is decreased expression of Le^x in esophageal adenocarcinomas that arise in Barrett's epithelium (10). In the Barrett group there was a correlation between the progression from normal to metaplasia to dysplasia to adenocarcinoma and the degree of suppression of Le^x expression. The observed loss of Le^x expression may prove useful in following patients with Barrett's epithelium in evaluating progression toward a malignant process.

CONCLUSIONS

Results to date continue to support the conclusion that the prognosis is poorer when low-risk small ductal breast carcinomas are positive for extended Le^a-Le^x oligosaccharide. However some of the most recent analysis, which has been associated with technical difficulties, has clouded this interpretation. The more complete testing of this possibility will await the completion and analysis of our final set of data examining the occurrence of the different cell surface oligosaccharides on cells of low-risk breast ductal carcinomas.. The SIL and DAF cDNAs cloned in this research are candidates for essential factors in the synthesis of extended Le^a-Le^x in cancer cells

REFERENCES

1. McGuire, W.L. and G.M. Clark. 1992 Prognostic factors and treatment decisions in auxiliary node-negative breast cancer. *N. Engl. J. Med* 326: 1756-1761.
2. Ottesen, G.L., H.P. Graversen., M Blichert-Toft, K. Zedeler, and J. Andersen. 1992 Ductal carcinoma *in situ* of the female breast: Short-term results of a prospective nationwide study. *Amer. J. Surg. Pathol.* 16: 1183-1196.
3. Cox, D. R. 1972. Regression models and life tables. *J. Royal Soc. B* 34: 187-220.
4. Pettijohn, D., O. Pfenninger, J. Brown, R. Duke and L. Olsson. 1988. Tumorigenic human squamous cell lung cancer cells have cell surface carbohydrates that are absent from nontumorigenic cells. *Proc. Natl. Acad. Sci. USA* 85: 802-808.
5. Stranahan, P., R. Howard., O. Pfenninger, M. Cowen, M. Johnson, and D. Pettijohn. 1992. Mucin gel formed by tumorigenic squamous lung carcinoma cells has Le^a-X oligosaccharides and excludes antibodies from underlying cells. *Cancer Res.* 52: 2923-2930.
6. Stranahan, P., LaRoe, J., McCombs, R., Goldsmith, A., Rahim, I., Overland, M. and D. Pettijohn. 1996. Cell-cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins. *Glycoconjugate J.* 13: 741-747.
7. Koretz, K., S. Bruderlein, C. Henne and P. Moller. 1992. Decay-accelerating factor (DAF, CD55) in normal colorectal mucosa, adenocarcinomas and carcinomas. *Br. J. Cancer* 66: 810-814.

8. Hadam, M. 1989. Cluster Report CD55. In *Leucocyte Typing IV; White Cell Differentiation Antigens*. Eds. Knapp, W., Dorken, B., Gilks, W., Rieber, E., Schmid, R., Stein, H and von dem Borne, A. p 694-97. Oxford Univ. Press.
9. Cheung, N., Walter, E., Smith-Mensah, W., Rainoff, W., Tykocinski, M., and Medof, M. 1988. Decay-accelerating factor protects human tumor cells from complement-mediated cytotoxicity *in vitro*. *J. Clin. Invest.* 81, 1122-1128.
10. Engel, U, McCombs, R., Stranahan, P., Pettijohn, D., and Hage, E. 1997. Decrease in Lex expression in esophageal adenocarcinomas arising in Barrett's Epithelium. *Cancer Epidemiology, Biomarkers & Prevention*. 6, 245-248.

BIBLIOGRAPHY

Stranahan, P., LaRoe, J., McCombs, R., Goldsmith, A., Rahim, I., Overland, M. and D. Pettijohn. 1996. Cell-cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins. *Glycoconjugate J.* 13: 741-747.

Engel, U, McCombs, R., Stranahan, P., Pettijohn, D., and Hage, E. 1997. Decrease in Lex expression in esophageal adenocarcinomas arising in Barrett's Epithelium. *Cancer Epidemiology, Biomarkers & Prevention*. 6, 245-248.

Personnel supported by this grant 1996-97

David Pettijohn, Professor
Patricia Stranahan, Assistant Professor
Iffat Rahim, Research Associate
Ronda McCombs, Professional Research Assistant
Vern Shellman, Professional Research Assistant
David Well, Student Dishwasher
Doug Tucker, Student Assistant